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The 20S proteasome mediates the degradation of mouse and yeast ornithine decarboxylase in yeast cells

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Abstract

Ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines, is one of the most rapidly degraded proteins in mammalian cells. Recently it has been demonstrated that mammalian ODC is degraded in vitro by the 26S protease that contains the 20S proteasome as its catalytic core, in a reaction that does not require ubiquitin. Here, we show that yeast and mouse ODC are both rapidly degraded in yeast cells and that their degradation severely inhibited in a mutant yeast cell line defective in the chymotryptic activity of proteinase yscE, the yeast 20S proteasome. These results provide compelling genetic support to previous biochemical studies suggesting the involvement of the 20S proteasome in the degradation of ornithine decarboxylase.

Key words: Ornithine decarboxylase; Proteinase yscE; Proteasome; Protein degradation; Yeast

1. Introduction

The proteasome, a 20S multicatalytic proteinase complex found in all eukaryotic cells, is highly conserved during evolution from yeast to human [1–7]. This 700 kDa cylinder-shape proteolytic particle, that consists of a large number of typical low molecular mass subunits, exhibits various proteolytic activities specific to proteins and peptides [7–9]. The 20S proteasome was recently demonstrated as a constituent of an even larger proteolytic conglomerate, termed 26S protease [10–12]. While the explicit physiological role of the free 20S particle is still obscure, it has been demonstrated that the 26S protease is involved in the ATP-dependent degradation of ubiquinated proteins [13,14]. So far, only two physiologically short lived proteins, c-mos and ornithine decarboxylase (ODC), have been demonstrated as specific substrates of the 26S protease in vitro [15–17]. Interestingly, while the degradation of the mos protein requires ubiquitin [16], that of ODC is ubiquitin independent [15,17]. However, the involvement of the 20S proteasome, or the 26S protease in the degradation of specific physiologically short-lived proteins in intact cells was not yet demonstrated.

Recently, a mutant yeast cell line, that is defective in the chymotryptic activity of the yeast 20S proteasome (proteinase yscE [18]) was isolated. Accumulation of the bulk of ubiquinated proteins in these mutant cells [18], and their inability to degrade β-galactosidase derivatives that are substrates of the ubiquitin-dependent, N-end rule pathway [19,20], indicate the involvement of the yeast proteasome in the ubiquitin-dependent proteolytic pathway. Here, using the yscE mutant cells, we demonstrate that the yeast proteasome is also involved in the in vivo degradation of ornithine decarboxylase.

2. Materials and methods

2.1. Cloning of yeast ODC DNA

Two synthetic oligonucleotides complementary to amino acids 111 to 118 and 407 to 415 of *Saccharomyces cerevisiae* ODC [21], were used as opposing primers in a polymerase chain reaction (PCR). The resulting 912 bp DNA fragment was used as an hybridization probe for screening a library prepared from *Eco*RI digested yeast genomic DNA. A 3 kb *Eco*RI fragment, from a positively hybridizing phage, was subcloned into the Bluescript plasmid (Stratagene). Restriction and sequence analysis demonstrated its identity to the previously isolated 3 kb *Eco*RI fragment that contains the yeast ODC gene [21].

2.2. Strains

Yeast strains used were: WCG4a (*MATa ura3 leu2-3,112 his3-11,15*) or WCG4-1-1a (*MATa ura3 leu2-3,112 his3-11,15 pre1-1*). To generate an ODC-deficient variant, cells representing these two stains were transformed with a 3 kb ODC fragment in which a 1 kb *Cla*I–*Bsm*I segment encompassing amino acids 147–466, and part of the 3′ noncoding region, was replaced by a 1.1 kb fragment containing a functional URA3 gene. Colonies that grew in the absence of uracil were selected and demonstrated to lack ODC activity. The two ODC deficient cell lines were used in the presented studies.

2.3. Expression of mouse and yeast ODC proteins in yeast cells

Mouse ODC cDNAs encoding the wild-type enzyme and the previously described stable carboxyl-terminal deletion mutant, Del-6 [22], (cloned in Bluescript between the *Eco*RI (5′) and *Bam*HI (3′) sites) were

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digested with *Eco*RV and ligated to *Bam*HI linkers. The resulting cDNA fragments, flanked by *Bam*HI sites, were ligated to the *Bgl*II site of the yeast expression vector pKV49 [23]. The 3 kb yeast ODC DNA fragment was digested with *Hin*dIII (located 177 nucleotides upstream to the translation start site) and with *Ssp*I (located 517 nucleotides downstream to the stop codon). The resulting 2.1 kb fragment was blunted with the klenow fragment of DNA polymerase-I, ligated to *Bam*HI linkers, and cloned into the *Bgl*II site of the pKV49 expression vector. Wild-type WCG4a cells and their isogenic WCG4a pre1-1a cells, that are defective in the chymotryptic activity of proteinase yscE, were transformed with the above described expression constructs. Since in these constructs the various ODCs were cloned under the regulation of the phosphoglycerate kinase (PGK) promoter, whose upstream activating sequences were replaced by those of the GAL-10 promoter, expression is induced by galactose and completely repressed by glucose [23].

2.4. Pulse-chase experiments and immunoprecipitation analysis

A 5 ml culture (OD_{600} = 0.5) of yeast cells grown in a minimal medium containing galactose, was harvested by centrifugation and incubated in 0.5 ml of the same medium supplemented with 100 μCi [^{35}S]methionine. After 10 min of incubation, cells were harvested either immediately or following a chase period in a minimal medium containing glucose. The cells were resuspended in immunoprecipitation buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.1% Triton X-100, 0.1% SDS), and disrupted by vortexing with glass beads. The extract was cleared by centrifugation and the amount of labeled proteins was determined by TCA precipitation. Portions containing equal amount of radioactivity were subjected to immunoprecipitation with anti-mouse, or anti-yeast ODC sera, prepared in rabbit against the respective antigens that were produced in bacteria [24]. Immunoprecipitated material was resolved by electrophoresis in a 10% SDS-polyacrylamide gel, and visualized by autoradiography. Each experiment was performed at least three times.

3. Results

In order to directly monitor expression and metabolic stability of various ODC proteins in yeast cells, cDNAs encoding wild-type yeast ODC, wild-type mouse ODC, and the stable mouse carboxyl-terminal deletion mutant, Del-6, [22], were cloned into the yeast expression vector pKV49 [23]. The resulting constructs were used to transform wild-type yeast WCG4a cells. Transformed cells harboring each expression construct were grown in a minimal medium containing galactose, and labeled with [^{35}S]methionine. Cellular extracts prepared either immediately or following the indicated chase periods were subjected to immunoprecipitation analysis, using highly specific anti-mouse or anti-yeast ODC sera. All three ODC proteins were efficiently expressed in the transformed yeast cells (Fig. 1). Moreover, as observed in mammalian cells [25,26], wild-type mouse ODC was rapidly degraded, while the carboxyl-terminal deletion mutant, Del-6, was stable (Fig. 1). In agreement with the reported rapid decay of yeast ODC activity in yeast cells [27], the yeast ODC protein decayed rapidly (Fig. 1).

Subsequently, in order to determine whether the observed degradation of the wild-type mouse and yeast ODC proteins requires the functional integrity of the yeast proteasome, WCG4a pre1-1 cells that are deficient in the chymotryptic activity of the yeast proteasome [18], were transformed with the expression constructs encoding the two labile wild-type ODC proteins. Pulse-chase analysis revealed that in contrast to their rapid degradation in wild-type cells, both wild-type ODC proteins were dramatically stabilized in the pre 1-1 mutant cells (Fig. 2).

Fig. 1. Degradation of mouse and yeast ODC proteins in yeast cells. Wild-type yeast WCG4a cells were transformed with pKV49 based expression constructs harboring wild-type mouse ODC, its stable Del-6 mutant and wild-type yeast ODC DNA. Transformed cells were grown in medium containing galactose, and labeled for 10 min with [^{35}S]methionine. Cellular extracts prepared immediately, or after the indicated time of chase in glucose containing medium, were subjected to immunoprecipitation analysis using highly specific anti-mouse or anti-yeast ODC sera. Immunoprecipitated material was fractionated by electrophoresis in a 10% SDS-polyacrylamide gel, and visualized by autoradiography. The position of the immunoprecipitated ODC proteins is indicated by arrowheads.

4. Discussion

We demonstrate here that yeast and mouse ODC, are rapidly degraded in wild-type yeast cells, but are dramatically stabilized in yeast cells harboring a mutant proteasome (yscE proteinase) which is defective in its chymotryptic activity.

We show here that as demonstrated previously in mammalian cells, also in yeast cells mouse ODC is a highly unstable protein. Moreover, as in mammalian cells, also in yeast cells ODC degradation requires the carboxyl-terminal destabilizing signal [22,28]. Interestingly, yeast ODC, which shares significant homology with the mouse enzyme, but lacks the carboxyl-terminal destabilizing segment, is rapidly degraded in yeast cells. This suggests that the recognition of yeast ODC by the yeast cellular proteolytic machinery is mediated by a recognition signal distinct from that of the mouse enzyme.

The degradation of the unstable wild-type mouse and yeast ODC proteins were directly tested in the WCG4a pre1-1 mutant that is defective in the chymotryptic activity of the yeast proteasome. Our results clearly demon-

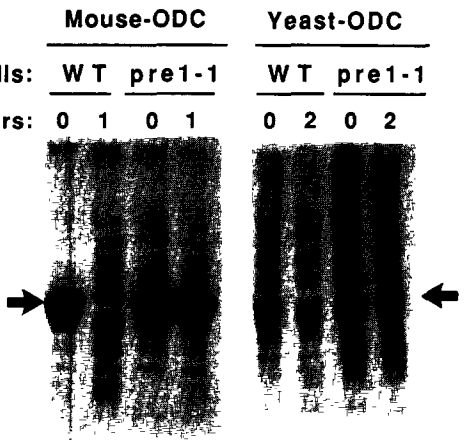

Fig. 2. Degradation of mouse and yeast ODC in wild-type yeast and in the pre1-1 mutant cells defective in the chymotryptic activity of proteinase yscE. Cells were grown in the presence of galactose and labeled for 10 min with [^{35}S]methionine. Cellular extracts were prepared either immediately, or following the indicated chase periods in glucose containing medium, and subjected to immunoprecipitation analysis as described in the legend to Fig. 1. The position of the ODC proteins is indicated by arrows.

strate that despite the difference in the signal that mediates their recognition, the degradation of both ODC proteins was dramatically impaired in the mutant strain. Based on these results, we conclude that the 20S proteasome is involved in the degradation of ODC in yeast cells. Recently, multiple forms of the mammalian 20S proteasome and the 26S protease were demonstrated [29]. Therefore, it could not be presently concluded, whether these two ODC proteins are recognized and degraded by the same 20S proteolytic particle, or by dissimilar proteasomes.

Recent studies have illustrated that the yeast proteasome (proteinase yscE) is involved in the degradation of ubiquinated proteins derived from different ubiquitin-dependent proteolytic pathways [18–20]. As we present here, the proteasome is also involved in the degradation of ODC, a process shown previously to be ubiquitin independent in mammalian cells [17,25,26]. Interestingly, studies in vitro revealed that in mammalian cells the 26S protease, not the free 20S proteasome, is involved in the degradation of ubiquinated proteins [10–14], and in the ubiquitin independent degradation of ODC [15,17]. It will be challenging to determine whether, in yeast, the 20S proteasome is directly responsible for the degradation of ubiquinated proteins and of ODC or, whether as in mammalian cells, it exerts its proteolytic role as a constituent of a larger more complex proteolytic conglomerate.

The 20S proteasome has been shown to exhibit three distinct proteolytic activities, trypsin-like, chymotrypsin-like and peptidyl-glutamyl-peptide hydrolyzing activity. Our present study demonstrates that a lesion in the chymotryptic activity severely inhibits ODCs degradation. It will be of interest to determine whether the other endopeptidase functions are also required for efficient degradation of ODC. Recently, another mutant cell line was isolated, showing deficiency in the peptidyl-glutamyl-peptide hydrolyzing activity [30]. In contrast to the pre1-1 mutants used in the present study, which accumulate ubiquitin-protein conjugates under stress conditions, this pre4-1 mutant exhibits no stress dependent phenotype. This difference emphasizes the interest in testing the degradation of ODC in the pre4-1 mutant, and in additional mutants of the yeast proteasome.

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